Application of real-time PCR and melting curve analysis in rapid Diego blood group genotyping

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The paucity of appropriate reagents for serologic typing of the Diego blood group antigens has prompted the development of a real-time PCR and melting curve analysis for Diego blood group genotyping. In this study, we phenotyped 4326 donor blood samples for Di^a using semiautomated equipment. All 157 Di(a+) samples were then genotyped by PCR using sequence-specific primers (PCR-SSP) for *DI*02* because of anti-Di^b scarcity. Of the 4326 samples, we simultaneously tested 160 samples for Di^a and Di^b by serology, and for *DI*01* and *DI*02* by PCR-SSP and by real-time PCR. We used the same primers for Diego genotyping by real-time PCR and PCR-SSP. Melting curve profiles obtained using the dissociation software of the real-time PCR apparatus enabled the discrimination of Diego alleles. Of the total samples tested, 4169 blood donors, 96.4 percent (95% confidence interval [CI], 95.8–96.9%), were homozygous for *DI*02* and 157, 3.6 percent (95% CI, 3.1–4.2%), were heterozygous *DI*01/02*. No blood donor was found to be homozygous for *DI*01* in this study. The calculated *DI*01* and *DI*02* allele frequencies were 0.0181 (95% CI, 0.0173–0.0189) and 0.9819 (95% CI, 0.9791–0.9847), respectively, showing a good fit for the Hardy-Weinberg equilibrium. There was full concordance among Diego phenotype results and Diego genotype results by PCR-SSP and real-time PCR. *DI*01* and *DI*02* allele determination with SYBR Green I and thermal cycler technology are useful methods for Diego determination. The real-time PCR with SYBR Green I melting temperature protocol can be used as a rapid screening tool for *DI*01* and *DI*02* blood group genotyping. ***Immunohematology* 2010;26:66–70.**

Key Words: blood donors, genotyping, real time, PCR, blood groups, Diego blood group, population, gene frequency

The Diego blood group system was named after identifying a new antibody, anti-Di^a, which caused HDN resulting in the death of a Venezuelan newborn in 1955.[1,2] Anti-Di^b was described in 1967 in two Mexicans by Thompson et al.[3] Anti-Di^a and -Di^b are clinically relevant. Both have been implicated in transfusion reactions and in HDN.[4–6]

The Diego blood group system comprises 21 antigens, and Di^a and Di^b are the most clinically significant.[7] Although Di^b is present in virtually all populations, Di^a incidence varies substantially worldwide. It is found in 7 to 54 percent of South American Indians, 5 to 8 percent of Asians (Chinese, Korean, Japanese), and 14.7 percent of Mexican Americans, whereas it is rare in Whites and Blacks (0.01%).[3,8,9]

Di^a and Di^b are carried on band 3 protein. The single *SLC4A1* gene (solute carrier family 4, anion exchanger, member 1) controls band 3 expression. This gene extends on an 18-kilobase (kb) genomic DNA, maps to chromosome 17q12–q21, and consists of 20 exons.[10] The *DI*01* and *DI*02* alleles code for the antithetical Di^a and Di^b antigens, respectively.[11]

*DI*01* and *DI*02* polymorphism is determined by a T>C nucleotide substitution at position +2561 in exon 19 of the *SLC4A1* gene, changing the leucine at amino acid position 854 to a proline in the band 3 protein.[12] No healthy individual with a Diego null phenotype has been reported, reflecting the functional importance of band 3. Although no Di(a–b–) subjects have been recognized by serologic testing, Alloisio et al.[13] described one individual who is homozygous for a band 3 mutation, Va1488Met (band 3 Coimbra), that results in almost complete deficiency of band 3. At the clinical level, Diego blood group antigens are of considerable importance in relation to their role in transfusion reactions and HDN. The Brazilian population is composed of a highly mixed ancestry, with an incidence of 1.3 percent of Di(a+) blood donors.[14] Consequently, multiply transfused individuals can have anti-Di^a or rarely anti-Di^b.[14–16] Furthermore, commercial anti-Di^a for serologic testing is scarce, and there is no available commercial anti-Di^b for routine use. The aim of this study was therefore to describe a DNA-based typing method that allows blood samples to be tested for *DI*01* and *DI*02* using a PCR real-time method.

Material and Methods

This is a prospective study performed at Fundação Pró-Sangue/Hemocentro, São Paulo, Brazil. A total of 4326 venous blood samples from unrelated Brazilian volunteer blood donors were collected in EDTA and tested for Di^a by hemagglutination. Every Di(a+) blood sample was then tested for Di^b by PCR using sequence-specific primers (PCR-SSP) owing to anti-Di^b scarcity. We then performed real-time PCR validation for Diego genotyping. Finally, we performed Diego analysis in 160 of 4326 blood samples for Di^a and Di^b by serologic studies, PCR-SSP, and real-time PCR simultaneously. The results were interpreted blinded from the serologic results.

Di^a phenotyping was performed using 50 µL of anti-Di^a (DiaMed AG, Cressier-sur-Morat, Switzerland) and 25 µL of a 1% RBC suspension dispensed into a microplate using semiautomated equipment (Megaflex-TECAN, TECAN AG, Hombrechtikon, Switzerland). After incubation for 30 minutes at 37°C, the microplates were centrifuged at 468*g* for 15 seconds. The microplates were then washed three times

with saline solution and centrifuged at 468*g* for 15 seconds. Fifty µL of antihuman globulin (AHG) serum (DiaMed Latino America, Lagoa Santa, Brazil) was added to each well; the microplates were centrifuged at 468*g* for 15 seconds, and read immediately. Di^b phenotyping was performed in gel cards (DiaMed Latino America) using anti-Di^b previously identified in a patient. The donor's RBCs were washed three times in 0.9% saline solution and suspended in LISS (ID-Diluent 2, DiaMed Latino America) to a final 0.8% suspension. In a microtube of the LISS/AHG ID card (DiaMed Latino America), 50 µL of 0.8% donor RBCs and 25 µL of patient's serum were dispensed and incubated at 37°C for 15 minutes in an appropriate incubator (ID-Incubator 37SI, DiaMed AG). After incubation, the cards were centrifuged for 10 minutes in an appropriate centrifuge (ID-Centrifuge 24S, DiaMed AG). After centrifugation, the cards were examined for agglutination or hemolysis according to the manufacturer's instructions. Positive and negative known control samples were included in each batch for Diego phenotyping validation results.

DNA Extraction

Human genomic DNA was isolated from whole blood in duplicate, using a commercial DNA extraction kit (QIAamp DNA Blood Mini Kit, QIAGEN Science, Hilden, Germany). DNA was extracted from 200 µL of blood and eluted in 100 µL of buffer according to the manufacturer's recommendations. For all samples, a mean of 100 ng of DNA was obtained. DNA was stored at –20°C for long-term storage.

PCR-SSP

All samples were genotyped in duplicate for *DI*01* and *DI*02* alleles by the PCR-SSP method and real-time PCR simultaneously, using primers designed by Wu et al.[17] (Table 1) The F2/AR pair of primers detected the *DI*01* allele, and the BF/R pair detected *DI*02*. Primers amplifying a fragment of the human growth hormone (HGH) gene served as an internal control.[18,19] PCR-SSP was carried out in a final volume of 25 µL containing 100 ng of purified DNA, 1.5 µL of $MgCl_2$ (1.5 mM), 1 µL of dNTP mix (0.2 mM, Invitrogen, Carlsbad, CA), 1.5 U of *Taq* polymerase (Platinum Taq DNA Polymerase, Invitrogen, São Paulo, Brazil), 2.5 µL of Tris-HCl (10 mM, pH 8.3, 50 mM KCl), 0.3 µM of each forward and reverse primer, and 0.08 µM of internal control. Amplifications were programmed on the thermocycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany), at the following conditions: denaturation at 95°C for 5 minutes, then 30 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 90 seconds at 72°C.[17] The reactions were completed by an elongation step for 5 minutes at 72°C. PCR products were visualized in a 2% agarose gel stained with ethidium bromide under 100 V using photodocumentation equipment (Eagle-eye; Stratagene, La Jolla, CA).

Real-time PCR

The same two sets of primers used for *DI*01* and *DI*02* polymorphism detection by PCR-SSP were also used for real-time PCR. Real-time PCR was carried out in 0.1-mL strip tubes and Caps (Corbett Research, Mortlake, Australia) using the same primers used for the PCR-SSP method (Table 1). Each reaction contained 7.5 µL of 2x Quantitect SYBR Green PCR Master Mix (QIAGEN Science), 0.3 µM of each forward and reverse primer, 100 ng of genomic DNA, and nuclease-free water in a final volume of 15 µL. PCR amplifications and fluorescence detection were performed using Rotor Gene 3000 equipment (Corbett Research, Sydney, Australia). The PCR amplification profile was a 95°C enzyme activation step (10 minutes), followed by 35 cycles of 95°C denaturation (30 seconds), 60°C annealing (30 seconds), and 72°C extension (90 seconds). Melting curves were generated by monitoring the continuous decrease in fluorescence of the SYBR Green signal from 75° to 95°C at the end of each run. Data acquisition and analysis were handled by the Rotor Gene 6 software (Corbett Research). In each run, we included samples homozygous for *DI*01* and *DI*02* used in the validation process as control samples.

Statistical Analysis

Allele frequencies were calculated by direct gene counting, and the differences were analyzed by the χ^2 test using a 2 × 2 contingency table. All analyses were performed on Statistica software (SAS Institute, Cary, NC).

Results

Real-time PCR Validation

In the validation process of real-time PCR for *DI*01* and *DI*02*, we tested 62 reference samples from the Fundação Pró-Sangue, after which these samples were genotyped for *DI*01* and *DI*02* using PCR-SSP (Figure 1A) and by real-time PCR (Figure 1B) on two occasions. The results were interpreted blinded from the serologic results. We found a genotype-specific melting profile for *DI*01* and *DI*02*, with both amplifications performed in one single run. This step was critical for the optimization of our real-time PCR protocol

Table 1. Primers used for *DI* genotyping by PCR SSP and real-time PCR

Primer	Sequence (5′-3′)	Ref	Pair of primers	Gene detected	PCR Product (bp)
F2†	GTGCTGGGGTGTGATAGGC	(17)	F2/AR	*DI*01*	139
AR†	CAGGGCCAGGGAGGCCA				
BF†	GGTGGTGAAGTCCACGCC	(17)	BF/R	*DI*02*	129
R†	CCAGGCAGCCACTCACAC				
HGH-F	TGCCTTCCCAACCATTCCCTTA	(18)	HGHF/ HGHR	*HGH*	434
HGH-R	CCACTCACGGATTTCTGTTGTGTTTC				

†GenBank accession no. AC003043 for *SLC4A1*
F = indicates forward primer; R = reverse primer.

and for determination of the Tm (melting temperature at which double-strand DNA is broken down to single-strand DNA) for *DI*01* (86.94°C) and *DI*02* (88.04°C) (Figure 2). Of 62 samples tested, 49 were found to be homozygous for *DI*02*. Eleven were *DI*01/02*, and two were *DI*01/01*. Our results for *DI*01* and *DI*02* genotyping with real-time PCR were totally concordant with those obtained by PCR-SSP. The two samples with a *DI*01/01* genotype by PCR-SSP allowed us to establish and validate the detection of the *DI*01* homozygous allele by real-time PCR.

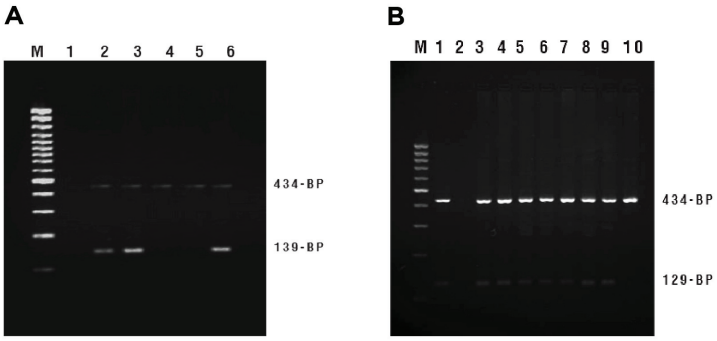

Fig. 1. PCR-SSP typing for *DI*01* and *DI*02* alleles. A 434-bp fragment of *HGH* was amplified as an internal control. Lane M shows a 100-bp ladder. (**A**) *DI*01* was determined by the presence of 139-bp fragment. From left to right: Blank (lane 1), *DI*01*-positive samples (lanes 2, 3), *DI*01*-negative (lanes 4, 5), *DI*01*-positive control (lane 6). (**B**) *DI*02* allele was determined by the presence of specific PCR product (129-bp).From left to right: *DI*02*-positive control (lane 1), Blank (lane 2), *DI*02*-positive samples (lanes 3, 4, 5, 6, 7, 8, 9) and negative control for *DI*02* (10).

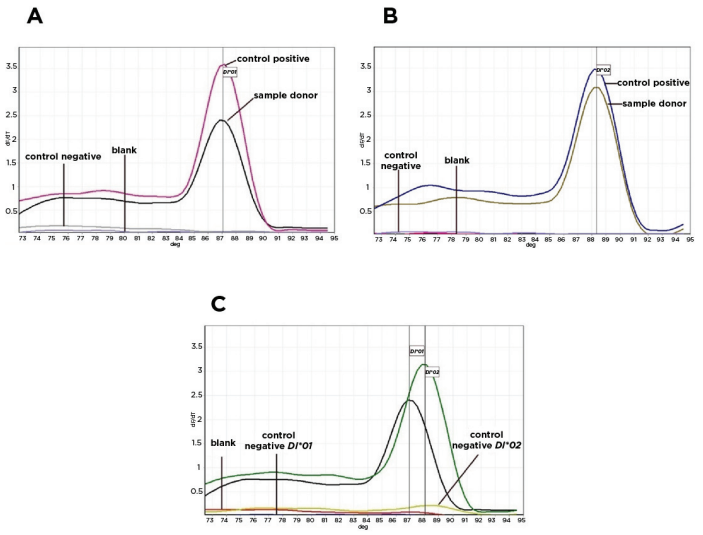

Fig. 2. Melting curve analysis of samples genotyped as (**A**) *DI*01/01*, (**B**) *DI*02/02*,and (**C**) *DI*01/02*. The *DI*01* and *DI*02* Tm were determined at 86.94° and 88.04°C, respectively.The arrows indicate controls (positive, negative) for *DI*01* and *DI*02*, blank, and sample donors tested.

Comparison of Phenotyping with Hemagglutination, PCR-SSP, and Real-time PCR for *DI*01* and *DI*02*

The sensitivity of our real-time PCR assay was verified by correctly identifying 160 samples for *DI*01* and *DI*02* by serology, PCR-SSP, and real-time PCR. Of these, 127 were found to be *DI*02/02* and 33 were *DI*01/02*. No single *DI*01/01* sample was identified in this series. All homozygous and heterozygous alleles have been distinguished by melting curve analyses. Although no *DI*01* homozygous individual was found in this series, we tested two *DI*01/01* samples in the validation step, and both were also identified by real-time PCR. Our results of Diego genotyping using real-time PCR were consistent and totally concordant with those results obtained using PCR-SSP. No discrepant results were observed among the three methodologies evaluated in our study.

Analysis of Diego Genotype and Allele Frequencies in Brazilian Blood Donors

Phenotype frequencies were estimated in Brazilian blood donors (Table 2). Among 4326 blood donors tested, 4169 (96.4%; 95% confidence interval [CI], 95.8–96.9%) were homozygous for *DI*02* and 157 (3.6%; 95% CI, 3.1–4.2%) were *DI*01/02* heterozygous. No blood donor was found to be homozygous for *DI*01* in this study (Table 2). We assumed that no blood donor presented the very rare band 3–deficient phenotype for Diego genotype and allele frequency calculation. The calculated *DI*01* and *DI*02* allele frequencies were 0.0181 (95% CI, 0.0173–0.0189) and 0.9819 (95% CI, 0.9791–0.9847), respectively, showing a good fit for the Hardy-Weinberg equilibrium (Table 3).

Table 2. Diego phenotype incidence in 4326 Brazilian blood donors

Phenotype	Number	%	95% CI
Di(a–b+)	4169	96.4	95.8–96.9
Di(a+b+)	157	3.6	3.1–4.2
Di(a+b–)	0	0	—
Total	4326	100	

χ^2 = 1.4776.
CI = confidence interval.

Table 3. Allele frequencies of *DI*01* and *DI*02* in 4326 Brazilian blood donors

Diego alleles	Allele frequency	95% CI
*DI*01*	0.0181	0.0173–0.0189
*DI*02*	0.9819	0.9791–0.9847

CI = confidence interval.

Discussion

In the last 10 years, the vigorous globalization process has made international travel and significant immigration extraordinarily common. Rare blood can be necessary in unexpected regions, causing an extra impact on blood services. Consequently, the Diego blood group system is of

clinical importance not only in Latin America but also in many other countries. Different approaches can be taken to deal with this situation.

Diego phenotyping has not been routinely performed in several blood services owing to the scarcity of commercial anti-Di^a. The situation is critical for Di^b, as there is no available commercial anti-Di^b. Therefore, whenever there is a suspicion of anti-Di^a or -Di^b, two problems are faced: one is to confirm the antibody specificity against the Diego antigens with appropriate controls (negative and positive), and the other is to find units negative for the antigen involved.

This need led us to search for an alternative approach for Diego blood group antigen testing and for the selection of blood units negative for Di^a or Di^b. Diego genotyping can be performed using the PCR-SSP method. However, PCR-SSP is a time-consuming assay as it requires post-PCR handling, and it has been shown to be inadequate for large-scale implementation.[20]

Wu et al.[17] described a PCR-SSP method for *DI*01* and *DI*02* genotyping that we used in this study. They tested different primer combinations and tried to use a single tube for PCR-SSP technique to identify *DI*01* and *DI*02* alleles in the same reaction. However, the results were disappointing, and they had to use two tubes, generating two separate products. Consequently, we used two separate protocols to test for *DI*01* and *DI*02* by PCR-SSP.

Real-time PCR for *DI*01* and *DI*02* allele determination can overcome these drawbacks because it allows specific amplification without post-PCR manipulations. Moreover, real-time PCR decreases the risk of error by the simple fact that it reduces the number of manual steps.[21] Recently, Polin et al.[22] also described the methodology for real-time PCR as an effective tool for blood group genotyping.

As labeled probes are costly when compared to real-time PCR with SYBR Green I, we decided to use the latter for cost reduction.[23] This decision also optimized our strategy for DNA analysis of other blood group systems, as we saw no need to purchase special primers.

The real-time PCR protocol that we developed allowed us to use the same primers we had been using for PCR-SSP in a universal cycling program, performing the same protocol to identify *DI*01* and *DI*02* alleles. The advantages of this real-time PCR method are the rapid performance and the detection of two alleles in the same run. The PCR assay revealed 100 percent specificity as assessed by comparison of genotype data to those generated by serologic typing. The Diego genotyping method described here, based on SYBR Green I by real-time PCR, can be used as a high-throughput discrimination of the *DI*01* and *DI*02* alleles (Figure 2). This approach takes advantage of the fluorescent property of SYBR Green I and of the melting curve analysis for the detection and discrimination of amplicons differing in length and nucleotide content.

One limitation of this study is that we were not able to detect the rare band 3 mutation called Coimbra, because we had not tested all samples by the three methods chosen. However, it would be highly unlikely to find one, as to date band 3 Coimbra mutation has been associated with anemia, and we only included nonanemic individuals (blood donors) in our study.[13]

Finally, we analyzed *DI*01* and *DI*02* genotype and allele frequencies in 4326 Brazilian blood donors. There are significant differences of *DI*01* and *DI*02* allele expression among world populations. The *DI*01* allele is considered rare in Whites,[24,25] but is characteristic in some Asians and in South American Indians with a gene frequency as high as 40 percent.[1,4] We detected Di^a in 3.6 percent of blood donors, with a *DI*01* gene frequency of 0.0181, indicating the complex ancestry miscegenation of the Brazilian population. This study was conducted in São Paulo State, located in southern Brazil, which is characterized by lower levels of African and higher degrees of European contributions when compared with other Brazilian groups.[26] Moreover, our results can be explained in part by the successive migratory waves from 1500 to the 20th century that contributed to the formation of the multiethnic highly admixed Brazilian population. This heterogeneity was documented in several genetic studies, which demonstrated a typical although nonuniform triethnic (European, African, and Amerindian) population gene pool.[27]

In conclusion, we developed a real-time PCR protocol for *DI*01* and *DI*02* genotyping that is feasible and easy to perform on a high-throughput scale. It can improve and facilitate anthropologic and epidemiologic studies on *DI*01* and *DI*02* allele determination.

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